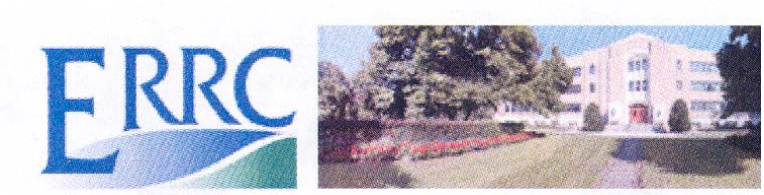

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**Title:** Numerical Analysis of the Growth of *Clostridium Perfringens* in Cooked Beef Under Isothermal and Dynamic Conditions

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# NUMERICAL ANALYSIS OF THE GROWTH OF *CLOSTRIDIUM PERFRINGENS* IN COOKED BEEF UNDER ISOTHERMAL AND DYNAMIC CONDITIONS[1]

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## ABSTRACT

*The main objective of this study was to develop a numerical technique to solve a set of biologically-based differential equations used to describe the growth behaviors of bacteria under isothermal conditions in food systems. A 4th-order Runge-Kutta method was incorporated to a computer program to solve these equations covering the entire range of bacterial growth, including lag, exponential, and stationary phases. The differential growth models were tested using the spores of* Clostridium perfringens *inoculated to ground beef and incubated under various isothermal conditions between 17-50C. Results of numerical analysis showed that the differential equations could accurately describe the growth of* C. perfringens *in cooked ground beef under isothermal conditions.*

*The differential equations were also used to estimate the growth of* C. perfringens *in cooked ground beef under four different dynamic temperature profiles: 2-square waves, exponential and linear cooling. In combination with a secondary kinetic model, the growth of* C. perfringens *in cooked ground beef under dynamic conditions was accurately estimated. Results showed the differences between the estimated and experimentally observed growth curves under dynamic conditions were generally less than 0.5 log (CFU/g). The methodology developed in this study can be a new approach for the food industry, food retailers and consumers, and regulatory agencies to predict and*

[1] Mention of a brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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*estimate the bacterial growth in cooked meat products under dynamic temperature abuse and deviation conditions.*

## INTRODUCTION

Growth of microorganisms in food systems is usually categorized into three different phases — lag, exponential, and stationary. Over the last few decades various mathematical models have been used to describe the sigmoidal trend of bacterial population growth under isothermal conditions. These models include Gompertz, logistic, and Baranyi models (Gibson *et al.* 1987; Baranyi *et al.* 1993a, b; McClure *et al.* 1994). Among these models commonly used in predictive microbiology, the Baranyi model was originally derived from the Michaelis-Menten and logistic kinetics, and therefore was considered more biologically-based than the modified Gompertz and logistic models. Each of these models is unique in its characteristics. The exponential phase described of a growth curve by the Baranyi model is more linear than the Gompertz and logistic models. The difference between the modified Gompertz and logistic models is in the symmetry with respect to the inflexion point (M), which is a point where the concavity of a curve changes. The logistic growth curves are symmetric with respect to the inflexion points (M), while the Gompertz curves are not. Although all these models have been widely used to describe the isothermal bacterial growth in food systems, obtaining them require applications of various nonlinear regression methods and highly sophisticated statistical software for curve fitting. The basic process of obtaining a growth model is (1) searching for a sigmoidal mathematical model, such as the Gompertz or logistic model; (2) modifying the model, usually accomplished by converting the independent valuable into the logarithm of bacterial counts; (3) using a statistical method, mostly nonlinear regression, to fit the data and obtain relevant parameters of the model. The traditional process is very convenient for obtaining a model, but such model usually lacks biological meaning, and it is necessary to rely on re-parameterization of the model to derive and define the biological meanings.

Juneja *et al.* (2001), Juneja and Marks (2002), and Juneja *et al.* (2003) proposed a new approach to describe the bacterial growth under isothermal conditions. This approach utilized a set of two differential equations to describe the population growth of pathogens in food systems:

$$\frac{dC_L}{dt} = -k_L C_L \qquad (1)$$

$$\frac{dC_D}{dt} = -\frac{dC_L}{dt} + k_D C_D \left(1 - \frac{C_D}{C_{max}}\right) \quad (2)$$

In Eq. (1) and (2), $C_L$ is the concentration of dormant bacterial cells, $C_D$ is the concentration of actively dividing cells, $C_{max}$ is the maximum cell concentration that bacteria can grow in the food system, $k_L$ is the rate at which bacteria leave the state of dormancy (or lag), and $k_D$ is the rate at which bacteria actively divide. Different from most empirical models (such as the modified Gompertz and logistic models) where the logarithm of bacterial counts or concentration is used, the unit for cell concentration in Eq. (1) - (2) is the original bacterial count, i.e., CFU per unit mass or volume.

The basic hypothesis for this model is that not all the initially inoculated cells/spores leave the lag phase at the same time. Instead, the process follows the 1st-order kinetics and is described in the first differential equation (Eq. 1). At time zero, all cells are presumed in the lag phase, and the cell concentration ($C_L$) is equal to the initial inoculum level. As soon as the bacterial cells exit the lag, they immediately begin to divide. The second differential equation (Eq. 2) governs the rate of bacterial division after the cells leave the lag phase. The initial condition for Eq. (2) is $C_D = 0$ at $t = 0$, i.e., there are no actively dividing cells at the time of inoculation. Combining Eq. (1) and (2), the mathematical equation describing the process of microbial division can be further written as

$$\frac{dC_D}{dt} = k_L C_L + k_D C_D \left(1 - \frac{C_D}{C_{max}}\right) \quad (3)$$

At any given moment, the total cell concentration (C) is the sum of the dormant and actively dividing cell concentrations:

$$C = C_L + C_D. \quad (4)$$

An analytical solution to Eq. (1) - (4) for a complete isothermal growth curve may not exist or is difficult to find. Therefore Juneja *et al.* (2001) and Juneja and Marks (2002) developed a partial solution to these equations by excluding the stationary phase from the growth curves. As a result, only partial growth curves were used to obtain the exponential growth rates and the lag phases in both studies conducted by Juneha *et al.* (2001) and Juneja and Marks

(2002). The authors still relied on nonlinear regression to obtain the growth parameters for the differential growth equations. This study represents a continuation of the previous effort and developed a methodology to solve the partial differential equations and use them to describe the entire bacterial growth process, covering from lag to stationary phases.

The growth models developed under isothermal conditions cannot be used directly for estimating bacterial growth under nonisothermal conditions. However, an important goal of predictive microbiology is to estimate the growth of pathogens in foods under dynamic temperature conditions. Several mathematical methods have been established to achieve this goal. These methods are primarily based on the modified Gompertz model (Van Impe *et al.* 1992, 1995; Huang 2003) and the Baranyi model (Baranyi and Roberts 1994; Bovill *et al.* 2000, 2001). From the data available in published literature, the method based on the modified Gompertz model seems more accurate than the one based on the Baranyi model in the dynamic analysis of bacterial growth.

The primary objective of this study was to develop a new approach using numerical techniques to solve the differential growth equations (Eq. 1-2) and use them to describe the entire bacterial growth curves under isothermal conditions. This research also aimed to apply numerical methods and the differential equations to estimate the bacterial growth under dynamically changing temperature conditions.

## MATERIALS AND METHODS

### Test Organisms and Sample Inoculation

Three different strains of *C. perfringens*, NCTC 8238 (Hobbs serotype 2), NCTC 8239 (Hobbs serotype 3), and ATCC 10388 (Hobbs serotype 13) were selected in this study to develop growth data completed with lag, exponential, and stationary phases. Spore crops of these strains were grown and harvested using procedures developed by Juneja *et al.* (1993). Each spore crop was washed twice, resuspended in sterile distilled water, and maintained at $\approx$ 4C until use.

Ground beef (93% lean), purchased from a local grocery store, was sterilized by ionizing $\gamma$-irradiation to a dose of 42 KGy at -30C using a $Cs^{137}$ source (Thayer *et al.* 1995). Three strains of bacterial spores with the same optical density were mixed to form a 10 mL cocktail and then inoculated to approximately 1500 g of ground beef. The bacteria and ground beef mixture was mixed twice (30 min each) in a Kitchen-Aid mixer (Model Max Watts 325) and a homogeneous distribution of spores was experimentally confirmed after the mixing. The final concentration of *C. perfringens* spores in ground beef was approximately 100 spores per gram of meat.

Inoculated ground beef was divided into 5 ± 0.02 g portions and packaged into plastic filter bags (12 × 19 cm, Model BagPage® BP 100, Topac Inc., Hingham, MA) and sealed at the vacuum of 15 mmHg. Samples were kept frozen (-20C) until used in the experiment.

### Isothermal Bacterial Growth

Inoculated frozen beef samples were thawed overnight in a refrigerator (≈ 4C). Samples in the plastic filter bags were first heat-shocked at 75C for 20 min to activate the spores and to inactivate any contaminating vegetative cells. The heat-shocked samples were briefly rinsed with running water (≈20C for 1-2 min) and placed into incubators maintained at 17, 25, 30, 36, 45, 47, and 50C, respectively. Samples from each incubation temperature were periodically removed from the incubators for determination of bacterial cell concentrations. Under isothermal conditions, *C. perfringens* spores could outgrow, germinate, and multiply. The incubation continued until a full growth curve, containing the lag, exponential, and stationary phases, could be developed. Experiments were conducted at least in triplicate to obtain the growth data under each isothermal temperature condition.

### Dynamic Bacterial Growth

To test the applicability of the differential growth models in estimating the bacterial growth under dynamic temperature conditions, inoculated samples were incubated under fluctuating temperature profiles. Samples were alternated between two incubators held under two different temperature conditions. At each exchange between the incubators, samples were taken and tested for bacterial counts. Two separate square-waved temperatures (30-45C and 45-36C) and were used.

Samples were also incubated under exponential and linear cooling conditions. Two continuously varying temperature profiles were arbitrarily chosen to test the methodology developed in this study. For both exponential and linear cooling temperature profiles, samples were placed in a circulating water bath (Model ESRB-7, Techne Inc., Princeton, NJ). For the exponential cooling tests, the water bath temperature was automatically controlled to change exponentially from 51C to 10C in 18 h. This temperature profile simulated a cooling condition with an internal temperature changing from 51C to 10C in 18 h under an ambient temperature of 0C. For the linear cooling tests, the water bath temperature was initially set at 51C and then changed at the rate of -0.1C/min. For both linear and exponential cooling tests, samples of the first 6 h were taken for analysis. The exponential cooling study was conducted in duplicate, while the linear cooling study in triplicate. Both cooling profiles were designed to test the adequacy of the differential equations and the numerical

method for estimating the bacterial growth under dynamic temperature conditions.

## Bacterial Counts

Samples removed from incubators were immediately diluted with equal volumes (5 mL) of 0.1% sterile peptone water. A rubber hammer was used to gently break and tenderize the meat samples in the plastic bags. The samples were then mixed in a MiniMix Stomacher (Model BagMixer® 100 W, Interscience Co., France) at the maximum speed for 12 min to completely homogenize the meat. After homogenization, a small volume (0.1-0.5 mL) of the liquid fraction was serially diluted with 0.1% sterile peptone-water and plated on Shahidi-Ferguson Perfringens (SFP) agar. After spread plating, each SFP agar plate was overlaid with approximately 10 mL of freshly prepared SFP agar. Upon solidification of SFP agar overlay, the plates were placed in an anaerobic chamber (Model Bactron IV, Sheldon Manufacturing Inc., Cornelius, OR) and incubated at 37C for 24-48 h under an atmosphere of $CO_2/N_2/H_2$ (10%:85%:5%). Typical perfringens colonies were counted and recorded.

## Numerical Analysis of Differential Equations

For a set of known experimental growth data obtained under an isothermal condition, the task of numerical analysis was to determine suitable values of $k_L$ and $k_D$ for Eq. (1)-(4). To simplify the calculation process, it is hypothesized that there exits an optimal value $\alpha$ such that $k_L$ is a linear function of $k_D$, or

$$k_L = \alpha k_D \tag{5}$$

A numerical iteration algorithm was designed to search for the most suitable values of $\alpha$ and $k_D$ for a set of experimental growth data. The least-squares method was used to determine the search criteria. The iteration started with an initial "guess" of $\alpha$ and $k_D$. After each iteration with "guessed" values of $\alpha$ and $k_D$, the computer program generated a growth curve. The computer-generated growth curve was then compared with the experimental growth data to calculate the sum of error squares (SES) using Eq. (6). In this equation, $C_j^E$ is the experimentally determined cell concentration at time $t_j$, $C_j^C$ is the computer-generated cell concentration at the corresponding time point, N is the total number of data points in the experimental set.

$$SES = \sum_{j=1}^{N} (C_j^C - C_j^E)^2 \quad (6)$$

The numerical iteration to search for $\alpha$ and $k_D$ would continue until a minimum value of SES for the input experimental growth data was found, indicating that the growth parameters for Eq. (1) and (2) best matching the actual growth data had been successfully obtained.

The 4th-order Runge-Kutta method (Chandra and Singh 1995) was used in the numerical iterations to solve the differential equations. A computer program was developed using Microsoft Visual Basic V6.0. This computer program required a set of actual growth data, including the initial and final concentrations. The original cell counts (CFU/g) were directly used in the computer program. After numerical analysis, a growth curve (described by the differential equations) that best fitted the experimental data was generated.

After the numerical iteration was completed, a pseudo-$R^2$ value was calculated using Eq. (7). Since the least-squares method was used to search for a growth curve that best fitted the experimental data, the errors would be evenly distributed around the experimental data points. Therefore, the higher the $R^2$ value, the more closely the differential equations found by the numerical algorithm matched the experimental data. The pseudo-$R^2$ value may not be a perfect indicator for the degree of fitness for the computer-generated growth curves, however, it was used in this study for comparative purposes.

$$R^2 = 1 - \frac{SES}{\sum_{j=1}^{N} (C_j^E - \bar{C})^2} \quad (7)$$

## Secondary Kinetic Model

To evaluate the temperature dependence of the population growth of *C. perfringens* in cooked beef, $k_D$ was correlated to temperature using a modified Ratkowsky equation (Zwietering *et al.* 1991):

$$k_D(T) = A(T - T_{min})^2 \{1 - \exp[B(T - T_{max})]\} \quad (8)$$

In Eq. (8), both A and B are coefficients, $T_{min}$ and $T_{max}$ are the theoretical minimum and maximum growth temperatures for *C. perfringens* in cooked ground beef. A Windows-based statistical package NCSS 2000 (Hintze 1999) was used to obtain the parameters of the equation using nonlinear regression.

### Estimation of Bacterial Growth Under Dynamic Temperatures

After Eq. (8) was available, the differential growth equations (Eq. (1)-(2)) were used to estimate the bacterial population growth under dynamic temperature conditions described previously. Again, the 4th-order Runge-Kutta method was used to simultaneously solve these equations. The temperature-dependent $k_D$ values were incorporated to the computer program to calculate the bacterial growth under dynamic temperature conditions.

## RESULTS AND DISCUSSION

### Selection of $\alpha$

In this research, it is assumed that there is linear relationship between $k_L$ and $k_D$ (Eq. 5), and $\alpha$ represents the interdependency between the two parameters. For a given set of experimental growth data, both $k_L$ and $k_D$ are unknown and must be determined by numerical analysis. Mathematically, there may be numerous combinations of $\alpha$ and $k_D$ values during numerical iterations to search for the best combination. With each selection of a $\alpha$ value, a corresponding $k_D$ could be found, and consequently a growth curve could be generated by the computer program. Figure 1 illustrates the effect of $\alpha$ on the result of numerical analysis for a set of experimental growth data.

Obviously lag phase and $k_D$ were both significantly affected by $\alpha$. As $\alpha$ increased from 0.0001 to 1, the lag phase of the computer-generated growth curve gradually decreased. At $\alpha = 1$, the computer-generated growth curve shows a very small lag phase, indicating that such a curve can be used to fit the growth data without lag phases. Generally, the $k_D$ values found by the computer program generally decreased with $\alpha$. However, it became independent of $\alpha$ when $\alpha$ was between 0.1 and 0.01 (Fig. 2). For *C. perfringens* in cooked ground beef, a $\alpha$ value of 0.01 was found suitable for fitting the majority of the experimental isothermal growth curves. Therefore this value was chosen in the computer program to search the best suitable $k_D$ values for all experimental growth curves.

### Isothermal Growth Curve Fitting

With $\alpha$ set to 0.01, all the experimental growth data were successfully analyzed to obtain the differential equations describing the growth behavior of *C. perfringens* in cooked ground beef. Numerical analysis showed the differential equations accurately matched the experimental data. The pseudo-$R^2$ values ranged between 0.963–0.999, indicating a close agreement between the computer-generated growth curves and the experimental data. As demonstrated

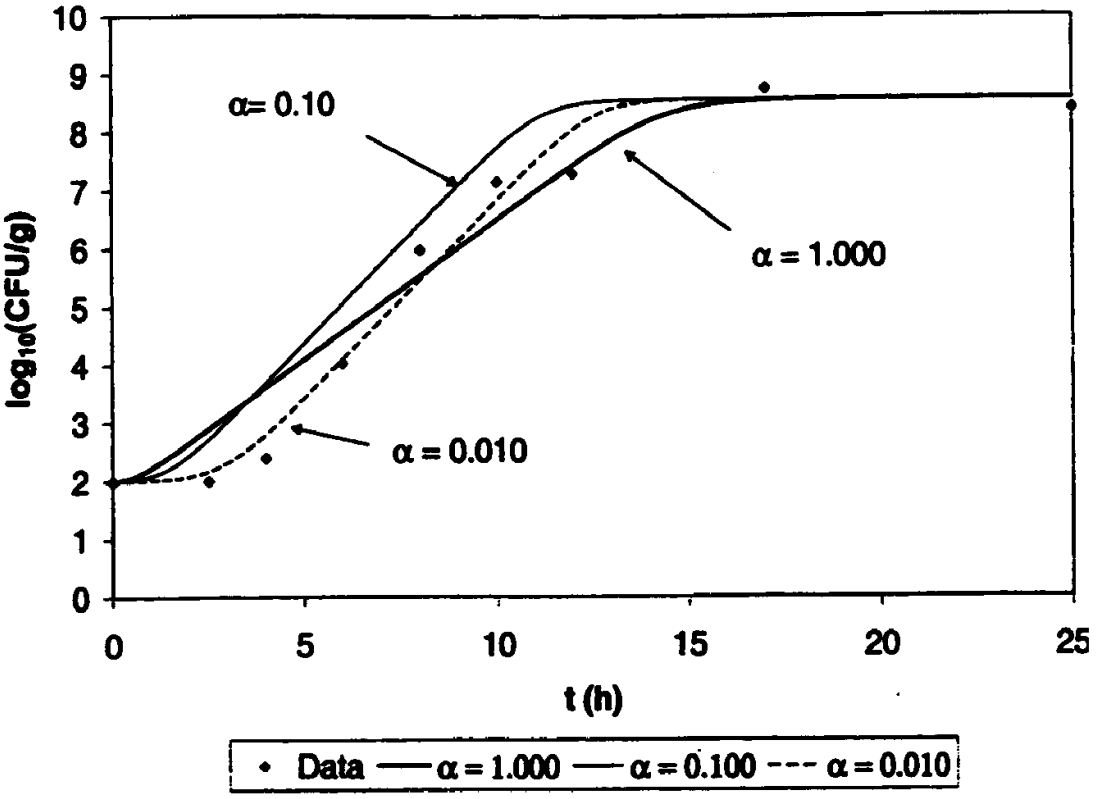


FIG. 1. EFFECT OF $\alpha$ ON THE SHAPE OF THE COMPUTER-GENERATED GROWTH CURVE FOR A SET OF EXPERIMENTAL GROWTH DATA

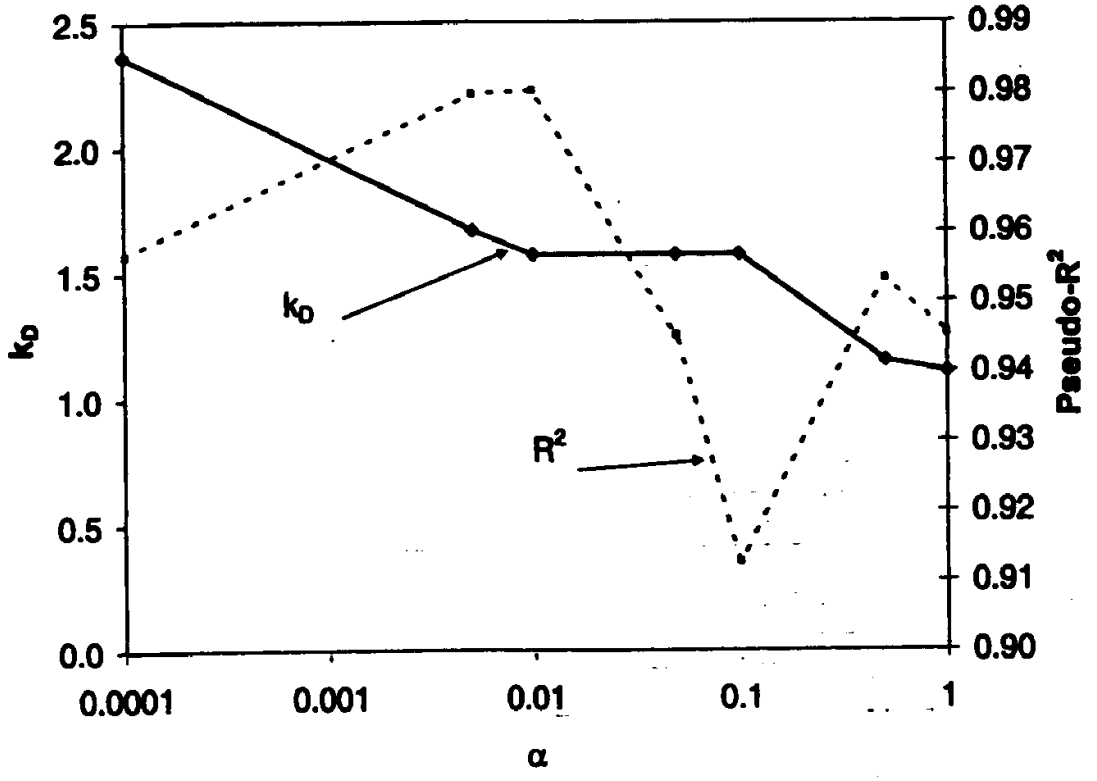


FIG. 2. EFFECT OF $\alpha$ ON $k_D$ AND PSEUDO-$R^2$

in Fig. 3, all of the three phases of microbial growth — lag, exponential, and stationary, are accurately described by the matching differential equations. A log-linear relationship between the cell concentration and incubation time can be clearly observed in the exponential phase, indicting that the differential equations can be used to depict the exponential growth nature of microbial division and multiplication.

## Secondary Growth Model

The slopes of the linear portions of growth curves, or $k_D$, were correlated to temperatures using a modified Ratkowsky equation (Fig. 4). The minimum and maximum growth temperatures estimated from the Ratkowsky model were 9.11 and 51.21C (Table 1). These values were in a close agreement with the values reported in the literature (Huang 2002; Juneja *et al.* 1999).

## Lag Phase

The growth curves fitted to experiment data show a general trend of the lag phase. However, the lag phase is not explicitly defined in the differential equations. Two methods can be used to define the lag phases of the growth curves. Buchanan and Solberg (1972) proposed an empirical method to determine the lag phase of an isothermal growth curve. They defined the lag phase ($\lambda_B$) as the time for the initial population to increase twofold. In the study reported by Juneja and Marks (2002), the lag phase ($\lambda_F$) of an isothermal growth curve was derived from the differential growth equations and is expressed in Eq. (9). In the current study, $k_D/k_L$ was 100 in Eq. (9). Therefore, the lag phase is inversely related to the rate constant, $k_D$. It was discovered that the lag phases determined from the empirical method (Buchanan and Solberg 1972) were almost identical to those determined from the formula method (Juneja and Marks 2002) (Fig. 5). The correlation coefficient between the two methods was 0.976, very close to 1. This is a clear indication that the definition of the lag phase by these two methods agreed very well.

$$\lambda_F = \frac{\ln\left(1+\frac{k_D}{k_L}\right)}{k_D} \tag{9}$$

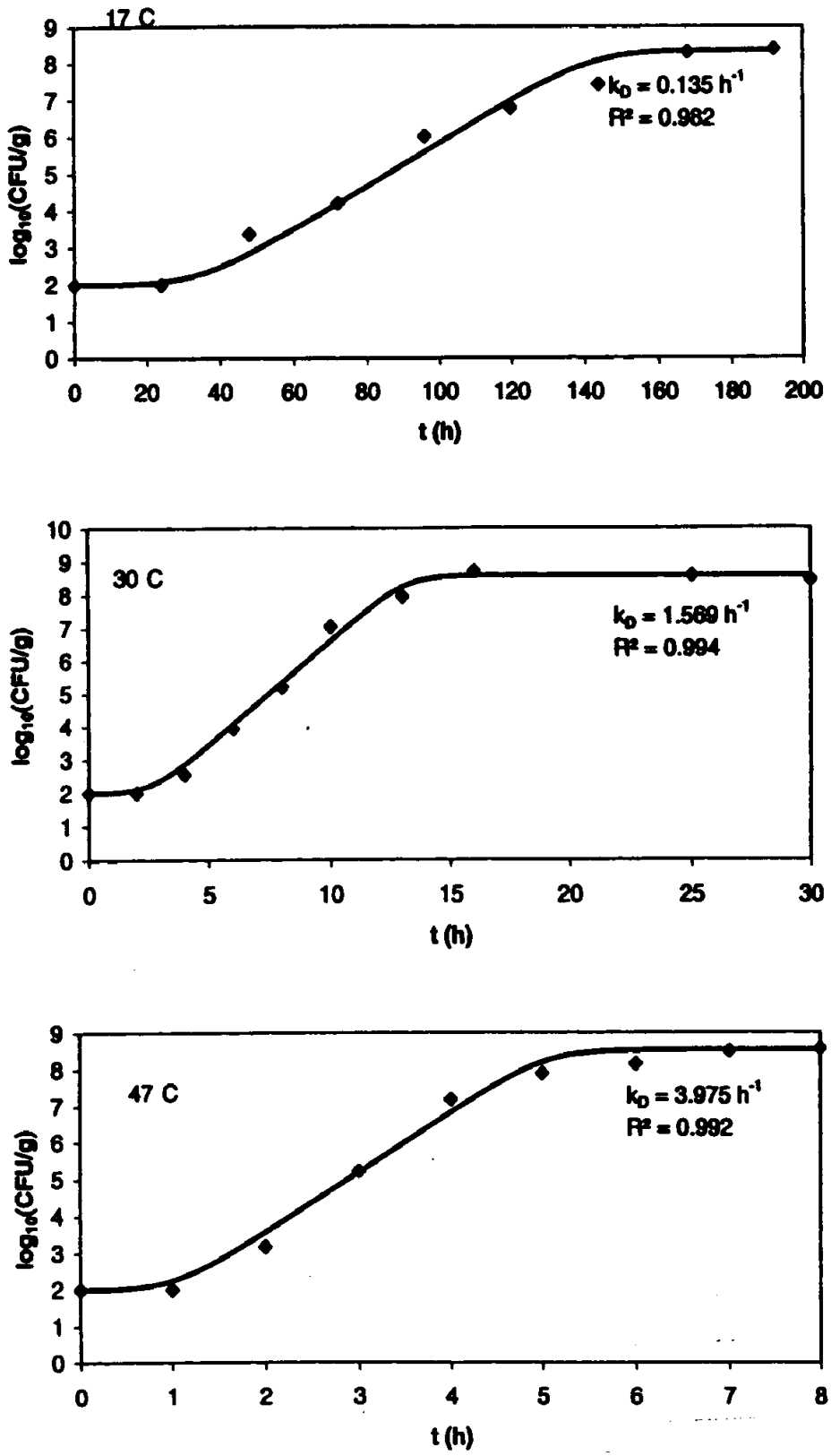


FIG. 3. REPRESENTATIVE GROWTH CURVES GENERATED BY NUMERICAL ANALYSIS FOR EXPERIMENTAL GROWTH DATA OBTAINED AT 17, 30, 47C, RESPECTIVELY

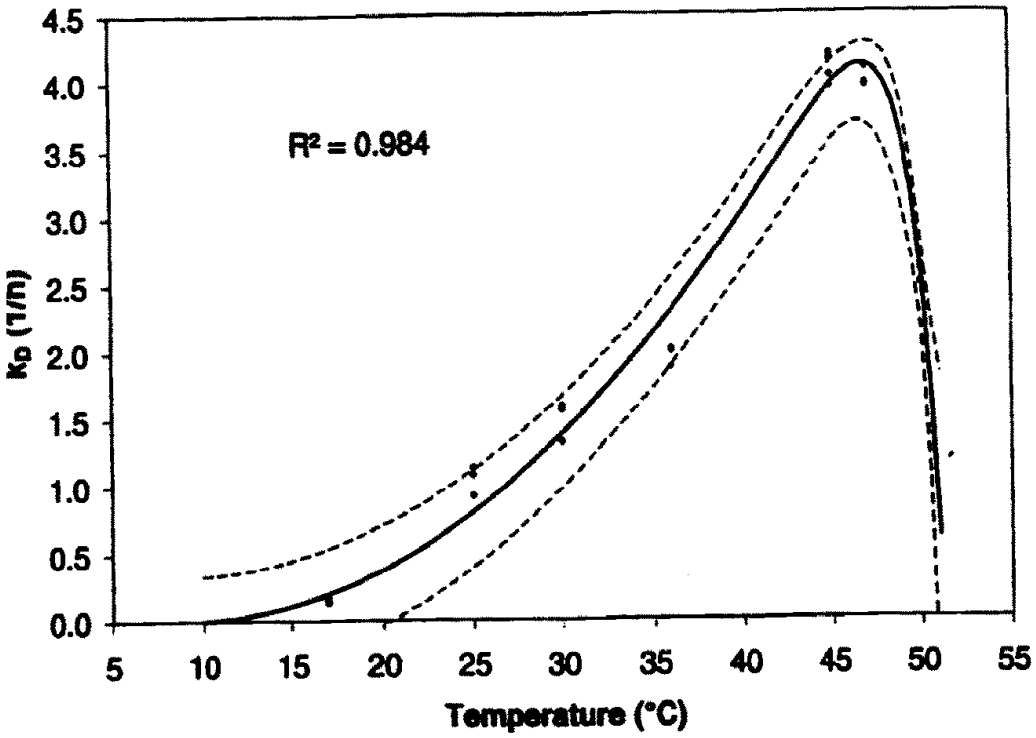


FIG. 4. DEPENDENCE OF $k_D$ ON TEMPERATURE AS DESCRIBED BY A MODIFIED RATKOWSKY EQUATION

Dotted lines represent the upper and lower prediction limits at a 95% confidence level.

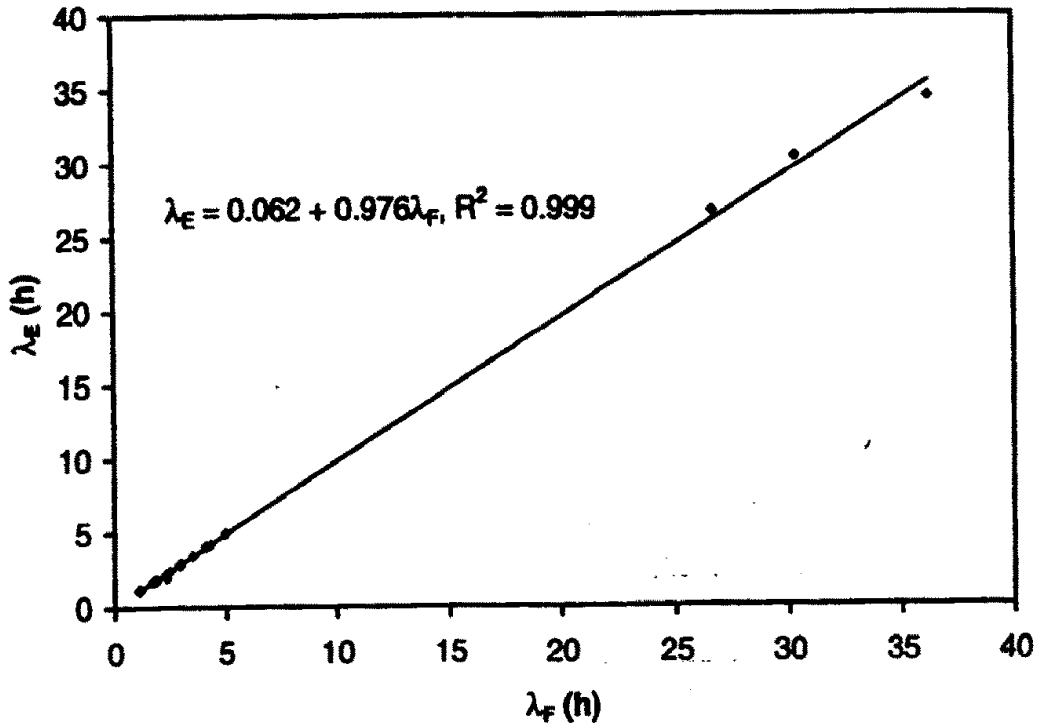


FIG. 5. THE CORRELATION BETWEEN THE LAG PHASES DEFINED BY THE EMPIRICAL METHOD (BUCHANAN AND SOLBERG 1972) AND THE FORMULA METHOD (JUNEJA AND MARKS 2002)

## Bacterial Growth Under Fluctuating Temperatures

With $k_D$ as a function of temperature (Eq. 8), the bacterial growth under fluctuating temperature conditions was estimated by simultaneously solving Eq. 2-4 using the 4th-order Runge-Kutta method. Figure 6A shows the result of the dynamic estimation of the growth of *C. perfringens* in cooked beef with incubation temperatures periodically fluctuating between 30-45C. Figure 6B represents the simulation result of the bacterial growth under temperatures fluctuating between 45-36C. For both simulation curves, the initial and maximum cell concentrations used in the numerical analysis were 2.0 and 8.31 log (CFU/g), respectively. The simulation result matched the experimental data closely, except at the early period (< 2 h) of the incubation process. Overall, the estimation errors were less than 0.5 log (CFU/g). There is an explanation for the discrepancy between the observed and computer-estimated growth data for *C. perfringens* during the early stage of incubation. For some reason, the population of this organism, both in spore and vegetative cell forms, usually experiences an initial decline when incubated. The degree of the initial decline in population is more severe at higher temperatures. This observation is called the "Phoenix phenomenon", explained in detail in a study reported by Shoemaker and Pierson (1976). This phenomenon usually occurs in the lag phase. The initial decline in the cell population does not mean that the organisms will eventually "die out". Instead, this organism starts to multiply rapidly after the lag phase. The differential equations developed in this study could not describe the "Phoenix phenomenon". The differential equations, however, can be used to estimate the potential bacterial growth as if the "Phoenix phenomenon" is not in effect. The results obtained from the differential equations represents a more conservative estimation of bacterial growth for *C. perfringens* in cooked beef during the early stage of incubation.

## Bacterial Growth Under Exponential and Linear Cooling Temperature Profiles

In conjunction with the secondary kinetic model with $k_D$ as a function of temperature (Table 1), the differential growth equations also can be used to estimate the bacterial growth under continuously changing temperature conditions. For *C. perfringens*, however, the vegetative cells may have to constantly adjust their metabolism to adapt to the environmental conditions during the initial stage of incubation. This would result in an increased lag time during the first few hours of incubation. Mathematically, the increased lag phase can be attributed to a reduced $k_L$ in Eq. (1), which controls the rate at which the inoculated cells leave the lag state or enter the state of active division and multiplication. Under dynamic conditions, the best choice of $k_L$ was experimentally determined as $0.0004k_D$. Figure 7 shows the results of the computer

estimation of bacterial growth processes during exponential and linear cooling. Except at the early stage of incubation ($t < 3$ h), the differences between the estimated and actual growth data were generally less than 0.5 log (CFU/g), indicating that the differential growth equations and the numerical method can be used to estimate bacterial growth under dynamic conditions. The discrepancy between the experimental and computer-estimated data in the early stage of dynamic growth also can be attributable to the "Phoenix phenomenon" mentioned previously.

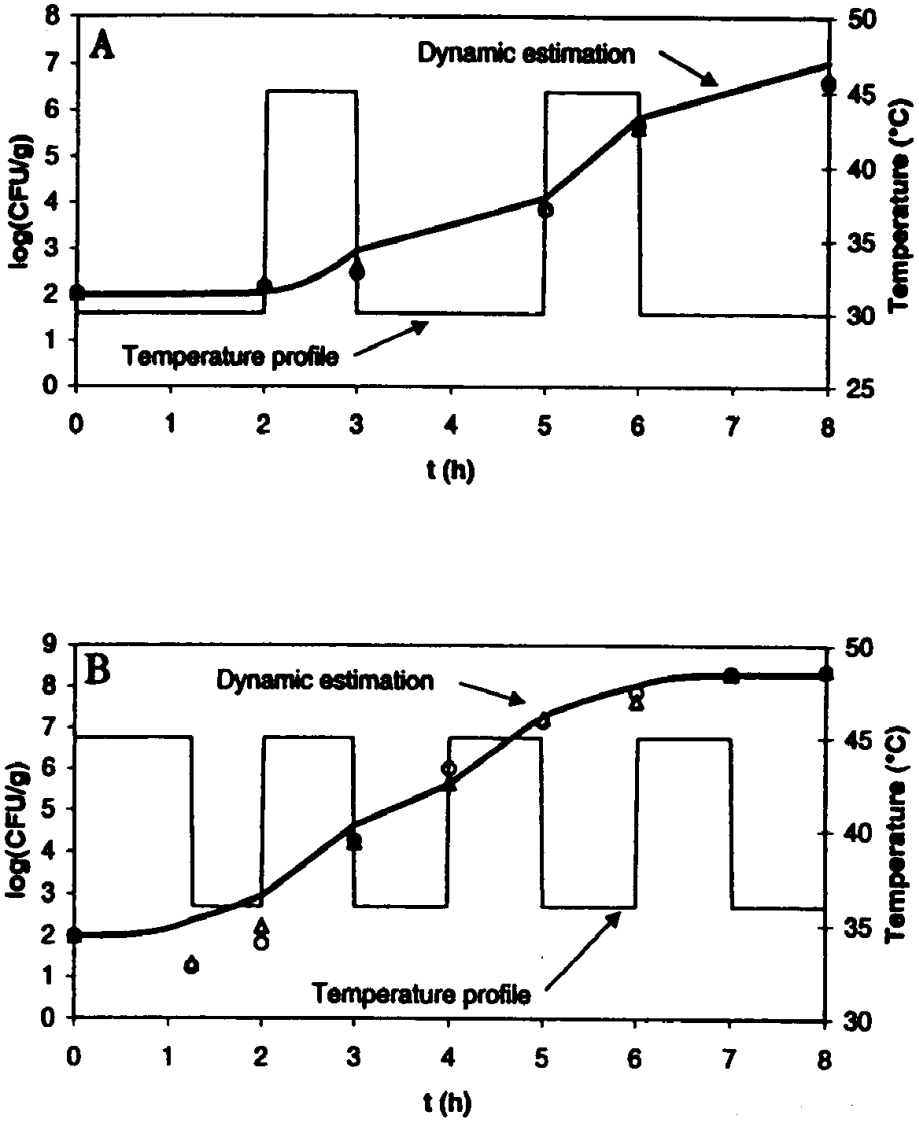


FIG. 6. DYNAMIC ESTIMATION OF THE GROWTH OF *C. PERFRINGENS* UNDER FLUCTUATING TEMPERATURE CONDITIONS

TABLE 1.
PARAMETERS OF THE MODIFIED RATKOWSKY EQUATION FOR $k_D$* SHOWN IN FIG. 4

| Parameter | Estimated Value | Asymptotic Standard Error |
|---|---|---|
| A | $3.187 \times 10^{-3}$ | $3.98 \times 10^{-4}$ |
| B | 0.5446 | 0.1711 |
| $T_{min}$ (°C) | 9.11 | 1.60 |
| $T_{max}$ (°C) | 51.21 | 0.33 |

$*k_D = A(T-T_{min})^2 \{1-\exp[B(T-T_{max})]\}$

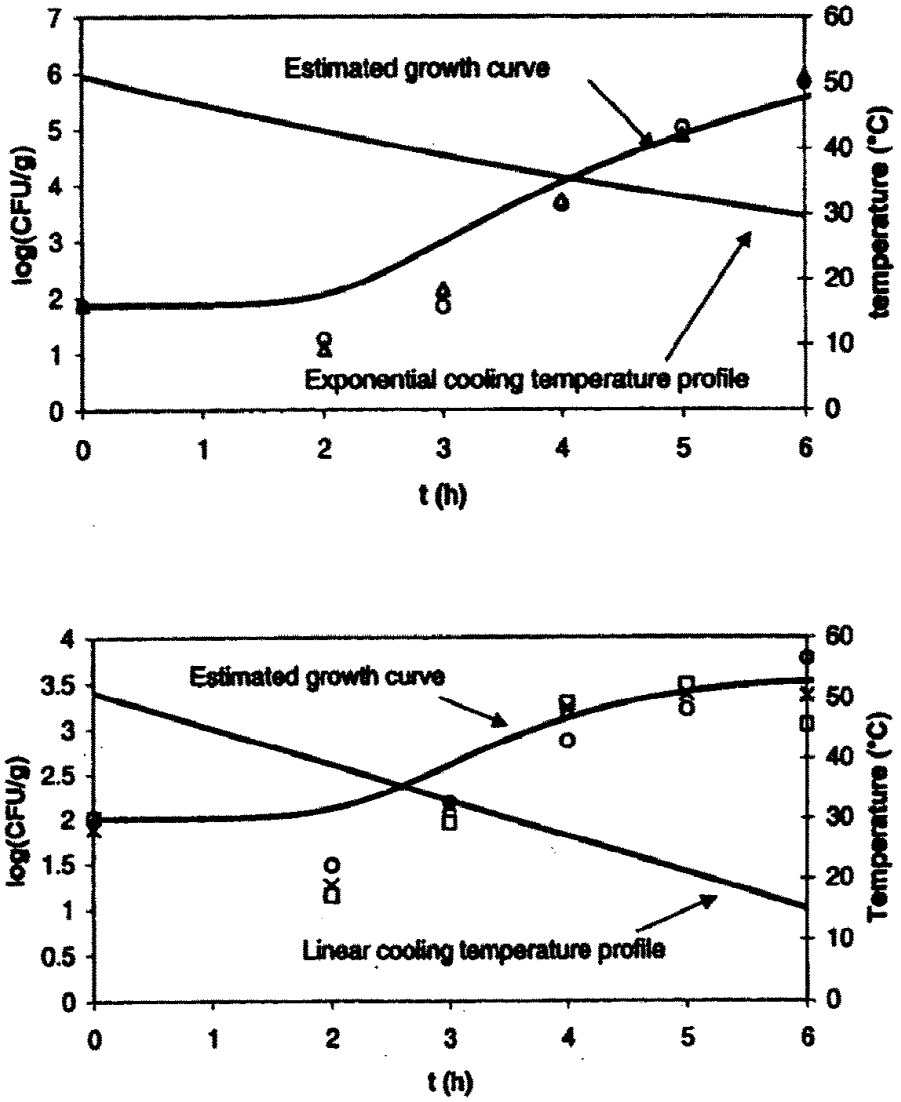


FIG. 7. DYNAMIC ESTIMATION OF THE GROWTH OF *C. PERFRINGENS* UNDER EXPONENTIAL AND LINEAR COOLING TEMPERATURE PROFILES

The numerical method used in this study to estimate the bacterial growth under dynamic conditions is similar in principle to the method developed by Van Impe *et al.* (1992, 1995), where the differential form of the Gompertz was used. The accuracy of the current method was also similar to the results obtained from the differential Gompertz equation (Huang 2003). Although both methods can be used to accurately estimate the dynamic bacterial growth, there is a difference between the two methods. The differential Gompertz equation was derived from the traditional modified Gompertz model, and therefore the method proposed by Van Impe *et al.* (1992, 1995) is essentially based on an empirical model. Both methods could accurately describe the bacterial growth under dynamic temperature conditions. The method developed in this study was not compared with the Baranyi's dynamic approach, however, due to the author's lack of understanding of its methodology.

## CONCLUSIONS

This study successfully validated the differential growth models proposed by Juneja *et al.* (2001) and Juneja and Marks (2002) using a numeric method in a computer simulation program. It was confirmed that the biologically-based differential equations were capable of depicting the entire isothermal growth curves, including the lag, exponential, and stationary phases. The results of numerical analysis of differential growth equations closely matched the experimental growth data for *C. perfringens* in cooked ground beef under isothermal conditions between 17-50C.

The differential equations also could be used to estimate the bacterial growth under dynamic temperature conditions. The secondary kinetic model, describing the temperature dependence of growth rates, can be used in conjunction with the differential equations to calculate the bacterial growth under dynamic conditions. Numerical analysis results of four temperature profiles matched closely with the experimental observations. Since the bacterial growth kinetics was integrated into the numerical algorithm, this computer program can be used to estimate and predict the growth of *C. perfringens* in cooked beef under any temperature conditions. Potentially it can be used to as a tool for real-time estimation and prediction of bacterial growth under temperature abuse and deviation conditions frequently encountered in the food industry and foodservice settings.

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